Hyperproduction of Erythropoietin in Nonanemic Lead-exposed Children

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Lead (Pb) poisoning has numerous effects on the erythropoietic system, but the precise mechanism whereby high dose exposure causes anemia is not entirely clear. We previously reported that Pb exposure is associated with depressed serum erythropoietin (EPO) in pregnant women residing in a Pb mining town and in a nonexposed town in Kosovo, Yugoslavia. In a prospective study, we tested the hypothesis that blood Pb concentration (BPb) may be associated with depressed EPO in children. BPb, hemoglobin (Hgb), and serum EPO were measured at ages 4.5, 6.5, and 9.5 years in 211, 178, and 234 children, respectively. At 4.5 years of age, mean BPbs were 38.9 and 9.0 µg/dl in the exposed and nonexposed towns, respectively; BPbs gradually declined to 28.2 and 6.5 µg/dl, respectively, by age 9.5 years. No differences were found in Hgb at any age. At age 4.5 years, a positive association between BPb and EPO ($\beta = 0.21$; $p = 0.0001$), controlled for Hgb, was found. The magnitude of this association declined to 0.11 at age 6.5 years ($p = 0.0103$) and 0.03 at age 9.5 years ($p = 0.39$). These results were confirmed using repeated measures analyses. We concluded that in Pb-exposed children, the maintenance of normal Hgb requires hyperproduction of EPO. With advancing age (and continuing exposure), this compensatory mechanism appears to be failing, suggesting a gradual loss of renal endocrine function due to Pb exposure. *Key words*: erythropoietin, hematopoiesis, lead, red cell survival. *Environ Health Perspect* 106:361–364 (1998). [Online 15 May 1998]
http://ehpnet1.niehs.nih.gov/docs/1998/106p361-364factor-litvak/abstract.html

High dose environmental lead (Pb) exposure induces anemia (*1–3*), the mechanism of which is not entirely clear. Elevated blood lead concentrations (BPbs) are associated with impaired heme synthesis (*4–6*), but even in severe Pb intoxication, elevated BPbs alone cannot account for the decrement in hemoglobin (Hgb) synthesis (*5*). Although porphyrins rise in Pb intoxication, the absolute depression of heme synthesis is inconsequential and cannot explain the fall in Hgb (*5,6*). Pb is known to have other effects on erythrocytes, including inhibition of pyrimidine-5′-nucleotidase activity (*7*), ineffective erythropoiesis (*8*), and shortened red cell survival (*9,10*), but these also do not completely explain the effects of Pb on Hgb concentration.

Several studies suggest important subclinical, perhaps preclinical, defects in hematopoiesis. Grandjean et al. (*11*) described delayed blood regeneration capacity in Pb-workers who had normal Hgb concentrations prior to blood donation. That report led us to hypothesize that Pb may inhibit the synthesis of erythropoietin (EPO), a glycoprotein hormone which regulates both steady-state and accelerated erythrocyte production. More than 90% of EPO is produced in the proximal renal tubule (*12,13*) where Pb accumulates; the remainder is produced in the liver. Indeed, we previously demonstrated that EPO is significantly depressed among pregnant women with moderately elevated BPbs (*14*).

Hu et al. (*15*) subsequently described a highly significant negative association between tibia (and patella) bone Pb concentration and Hgb among 119 nonanemic men from the Carpenters' Union; yet no association between BPb and Hgb was found. This report suggests that bone Pb may be a more important marker of ongoing Pb toxicity than BPb. The authors suggested that the observed associations may reflect inhibition of hematopoiesis through depression of EPO, with bone Pb serving as a proxy for kidney Pb (*15*).

As part of a long-term prospective study of environmental Pb exposure during infancy and childhood (*16–18*), we examined the associations between BPb and EPO at ages 4.5, 6.5, and 9.5 years. We found that children with elevated BPbs maintain normal Hgb, but require hyperproduction of EPO to do so. Our working hypothesis is that children with moderately elevated BPbs have shortened red cell survival, a phenomenon previously described only in children with Pb encephalopathy (*9*) and in Pb workers (*10*).

Methods

This study was conducted in two towns in Kosovo, Yugoslavia: Kosovska Mitrovica (K. Mitrovica), the site of a Pb mine, smelter, refinery, and battery plant; and Pristina, a relatively nonexposed town 25 miles to the south. Children were selected for follow-up from a previous prospective study of 1,502 pregnant women residing in these towns. Pregnancy outcomes (*19,20*) and childhood developmental outcomes have been previously described (*16–18*).

Subjects. In brief, 706 mother–infant pairs from the pregnancy study were invited to participate in a follow-up study involving repeated visits at 6-month intervals. Of these, the parents of 541 consented and brought their children to at least one visit. Of those who consented, 311 (53.5%), 267 (49.4%), and 260 (48.1%) participated in the visits at ages 4.5, 6.5, and 9.5 years, respectively. For the present study, whole blood for BPb and Hgb was available for 272, 201, and 234 children at each of these ages, respectively; sera for EPO analyses were available for 211, 178, and 234 children, respectively.

Laboratory analyses. At mid-pregnancy, delivery, and at each 6-month follow-up visit, venous blood samples were taken for the measurement of BPb (*21*), erythrocyte protoporphyrin [EP (*22*)], and Hgb. At each visit, additional sera were obtained and frozen immediately to create a serum bank, from which the current study is derived. Whole blood and serum samples were stored at 4°C and -20°C, respectively, and appropriately transported to Columbia University where all assays were performed. The laboratory participates in the BPb and EP quality control program of the Centers for Disease Control. During the study period relevant to this analysis, agreements with the quality control values for BPb and EP, measured by intraclass correlation coefficients, were both 0.99.

All available sera from the visits at ages 4.5, 6.5, and 9.5 years were analyzed in duplicate for EPO using a commercially available enzyme immunoassay (*23*). In our laboratory, the limit of detection of the assay was 0.6 mIU/ml, and the coefficient of variation for duplicate measures was 9.8%, 8.3%, and 7.9%, respectively, at ages 4.5, 6.5, and 9.5 years.

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This study was supported by grant ES 03460 from the National Institute of Environmental Health Sciences.
Received 26 January 1998; accepted 24 March 1998.

Statistical analysis. The association between BPb and EPO was examined using regression methods. Because the distributions of EPO and BPb are skewed, logarithmic transformations were used for these variables. All analyses were performed using SAS version 6.12 (SAS Institute, Cary, NC). First, we evaluated the association between BPb and EPO at each age using linear regression analysis, controlling for concurrent Hgb concentration. Concurrent Hgb was always controlled because it is the most important predictor of EPO (*13*). Second, we combined data from all ages and used repeated measures analysis (described below) to determine whether the associations between BPb and EPO, controlling for Hgb, changed over time.

The repeated measures analysis was based on 614 observations from 346 children with EPO, BPb, and Hgb measurements available for at least one time point (i.e., ages 4.5, 6.5, or 9.5 years). The 614 observations included 157 (45.5%) children who had measurements made at one time point (i.e., one age), 110 (31.8%) children with measurements from two time points, and 79 (22.8%) children with data from all three time points of interest. The repeated measures analysis was performed using the marginal linear model [GEE (*24*)], which takes into account the within-subject correlations between these variables; the details of this analysis and its findings are provided in the Appendix.

For the 15 subjects with missing values for BPb or Hgb at a specific time point, we used the following algorithm to substitute values. If BPb (or Hgb) measurements were available for both the 6 months prior to and the 6 months after the missing values, we substituted the mean of these measurements. If BPb (or Hgb) was available for only one of these time points, that value was substituted. Thus, substitutions were made for 12 of these 15 subjects.

Results

During the course of the 5-year period, mean BPb in each town declined by approximately 28% (Fig. 1A). Mean Hgb in each town remained constant and within the normal range (Fig. 1B). At ages 4.5, 6.5, and 9.5, BPbs ranged from 4.6 to 73.1, 3.1 to 71.7, and 2.3 to 58.1 µg/dl, respectively; hemoglobin concentrations ranged from 9.5 to 14.5, 10.7 to 15.1, and 9.6 to 15.3 g/dl, respectively; and EPO concentrations ranged from 2.4 to 40.4, 1.6 to 27.1, and 2.1 to 24.0 mU/ml, respectively. In Pristina, the mean EPO rose steadily over time from 5.6 to 8.8 mIU/ml (Fig. 1C). In K. Mitrovica, EPO rose between ages 4.6 and 6.5 years (from 7.8 to 9.7 mIU/ml) and then declined to 8.8 mIU/ml at age 9.5 years. The differences in EPO between towns were statistically significant at ages 4.5 (p = 0.0017) and 6.5 years (p = 0.02), with higher concentrations in K. Mitrovica.

Because Hgb is a strong determinant of EPO (*13*), EPO was examined within Hgb strata for each age (Fig. 2). Few children were anemic; there were 9, 1, and 1 children with Hgb below 11.0 g/dl at ages 4.5, 6.5, and 9.5 years, respectively. Within each Hgb stratum, EPO was higher in K. Mitrovica except at age 9.5 years, when the town differences diminished.

Associations between BPb and EPO, adjusted for Hgb and specific to each age, are shown in Table 1. As expected in each of these models, Hgb was inversely and significantly associated with EPO. Statistically significant associations between BPb and EPO were found at ages 4.5 and 6.5 years; however, the magnitude of the association at age 6.5 was approximately half that at age 4.5. At age 9.5 years, the association was not statistically significant and was approximately an order of magnitude less than that at age 4.5.

Results of a regression analysis using repeated measures were similar. At ages 4.5 and 6.5 years, BPb was significantly and positively associated with EPO (p<0.0001 and p = 0.0007, respectively). This association diminished at 9.5 years of age. Indeed, the difference in the association between BPb and EPO at ages 4.5 and 9.5 years was statistically significant (p = 0.0006), while that between ages 4.5 and 6.5 was not. To test whether the association between BPb and EPO varied by Hgb, we repeated all analyses including the appropriate interaction terms. Based on this GEE model and after adjustment for Hgb, a significant interaction between BPb and age was found, indicating that the association between EPO and BPb varied by age (see Appendix). Further analyses found no interaction of Hgb and age.

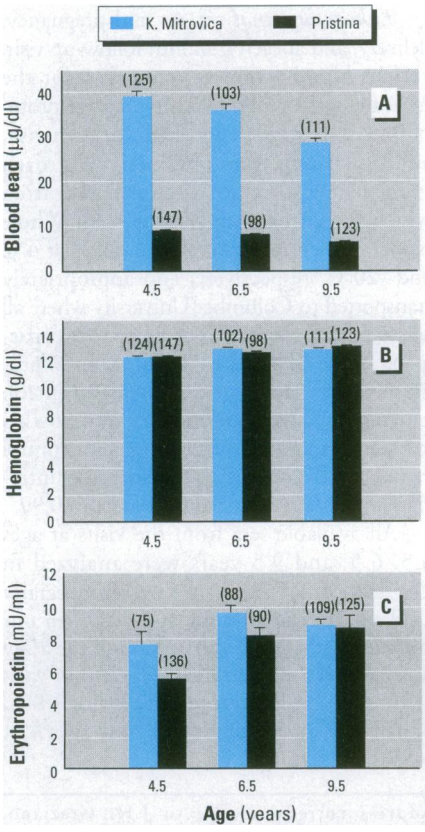

Figure 1. Mean blood lead (A), hemoglobin (B), and serum erythropoietin (C) at 4.5, 6.5, and 9.5 years of age in Kosovska Mitrovica (K. Mitrovica) and Pristina, Yugoslavia. Numbers in parentheses are the number of observations; the error bars reflect the standard error.

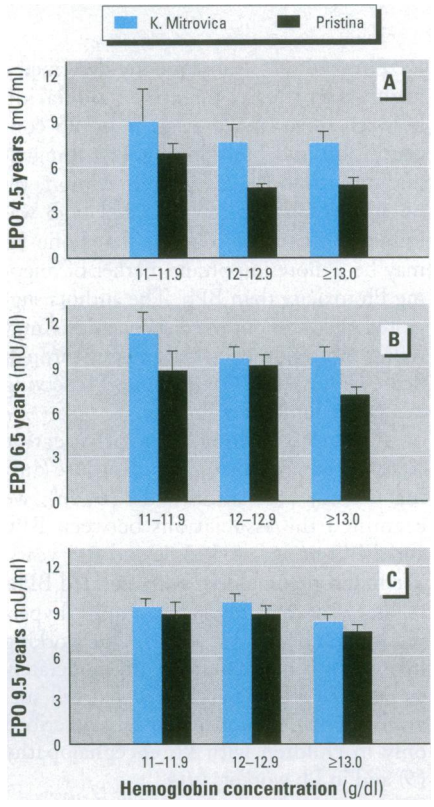

Figure 2. Mean serum erythropoietin (EPO) concentrations in children from Kosovska Mitrovica (K. Mitrovica) and Pristina, Yugoslavia stratified by hemoglobin concentration. At 4.5 (A) and 6.5 years of age (B), children in K. Mitrovica had significantly higher serum EPO. By 9.5 years of age (C), serum EPO concentrations were comparable. Error bars indicate standard error.

Table 1. Regression coefficients relating blood lead concentration to serum erythropoietin concentration for children 4.5, 6.5, and 9.5 years of age, residing in two towns in Kosovo, Yugoslavia

Age (years)	β[a]	SE[b]	p-Value
4.5	0.21	0.043	0.0001
6.5	0.11	0.041	0.0103
9.5	0.029	0.033	0.39

[a]Regression coefficient controlled for hemoglobin concentration.
[b]Standard error for the regression coefficient.

Discussion

We previously reported that Pb exposure was associated with depressed serum EPO in pregnant women (*14*). Our follow-up prospective study of their children provided a unique opportunity to longitudinally examine subtle hematologic effects of Pb exposure in a population whose lifetime exposure has been well characterized. In the current analyses, we observed that serum EPO, after adjustment for Hgb, was positively associated with BPb in children at ages 4.5 and 6.5 years. The findings imply that in nonanemic Pb-exposed children, increased erythrocyte production is required to maintain normal Hgb concentrations. There is strong evidence, discussed below, to support the conclusion that this compensatory mechanism is a response to Pb-induced shortened red cell survival. With age, however, the adjusted regression coefficients between BPb and EPO (Table 1) declined from 0.21 at 4.5 years (p = 0.0001) to 0.11 at 6.5 years (p = 0.0103), to 0.03 at age 9.5 years (p = 0.39). It is possible that this gradual decline in slope was due to the modest decline in mean BPb as children aged, i.e., that BPbs fell below a critical threshold for the putative Pb effect. However, our previous study in pregnant women (*14*) found depressed serum EPO in women whose BPbs were substantially lower than the children in the current study. Thus, it appears more likely that the decline in slope with age reflects a gradually decreasing ability to produce EPO. We also note, however, that the relationship between BPb and EPO is subtle because the observed serum EPO concentrations in this cohort were nearly all within the published age-related norms (*25*).

A review by Aub et al. in 1925 (*1*) concluded that although severe plumbism in man may involve "bone marrow failure," anemia was initially due to accelerated destruction of circulating red cells, a phenomenon first suggested in the 19th century (*26,27*). Supportive mechanistic studies revealed that patients with plumbism had red cell membrane defects which were associated with increased red cell mechanical fragility and decreased osmotic fragility (*1*). In animals, Pb-induced anemia was found to be reversed by removal of the spleen (*28*), which removes abnormal and/or aged red cells from circulation.

Numerous animal studies indicate that a two-step sequence of events is involved in Pb-induced anemia. For example, in rabbits acutely poisoned with Pb, peripheral red cell destruction was followed by active bone marrow hyperplasia (*29*), indicative (in retrospect) of active renal EPO production. With time, however, rabbit bone marrow activity declined, suggesting either failure of erythropoietic stem cells, a decline in renal EPO production, or both. The same temporal sequence has been described in children. Leikin and Eng (*9*) described shortened ^{51}Cr red cell survival in children with BPbs of 60–238 µg/dl. Among their patients whose Pb exposure was short in duration, turnover of ^{59}Fe in plasma was abnormally high, indicative of bone marrow hyperactivity; in those with long-term Pb exposure iron turnover was depressed, consistent with marrow hypoactivity.

Our finding that BPb was positively associated with EPO in children at ages 4.5 and 6.5 years is consistent with the first pathophysiologic step, i.e., peripheral red cell destruction, renal EPO production, and bone marrow hyperactivity. Our earlier finding that EPO is negatively associated with BPb in moderately anemic pregnant women [average age = 26 years (*14*)] is consistent with the second pathophysiologic step, i.e., bone marrow hypoactivity due to chronic renal, and possibly marrow, Pb toxicity. We speculate that the lack of association between BPb and EPO at age 9.5 years of age may represent a transitional period, possibly leading to insufficient EPO compensatory reserves later in life.

References and Notes

1. Aub JC, Fairhall LT, Minot AS, Reznikoff P. Lead poisoning. Medicine 4:1–250 (1925).
2. Baker EL Jr, Landrigan PJ, Barbour AG, Cox DH, Folland DS, Ligo RN, Throckmorton J. Occupational lead poisoning in the United States: clinical and biochemical findings related to blood lead levels. Br J Ind Med 36:314–322 (1979).
3. Schwartz J, Landrigan PJ, Baker EL Jr, Orenstein WA, von Lindern IH. Lead-induced anemia: dose–response relationships and evidence for a threshold. Am J Public Health 80:169–172 (1990).
4. Lichtman HC, Feldman F. *In vitro* pyrrol and porphyrin synthesis in lead poisoning and iron deficiency. J Clin Invest 42:830–839 (1963).
5. Piomelli S, Lamola AA, Poh-Fitzpatrick MB, Seaman C, Harber LC. Erythropoietic protoporphyria and lead

Appendix

We fitted a repeated measures model using the generalized estimating equations (GEE) methodology. This model allows for independent effects of age and blood lead concentration (BPb) at each age on erythropoietin (EPO) at each age. In all, 346 observations were available for this analysis (i.e., 346 measures of EPO). The regression model takes the form:

Regression model [for j = 1,2,3; i = 1,...n(= 346)]

$$\text{lgEPO}_{ij}=\gamma^{*}\text{Hgb}_{j}+(\alpha_0+\beta_0{}^{*}\text{lgBP}_{ij})I(a_{ij}=4.5)+(\alpha_1+\beta_1{}^{*}\text{lgBP}_{ij})I(a_{ij}=6.5)+(\alpha_2+\beta_2{}^{*}\text{lgBP}_{ij})I(a_{ij}=9.5)+\varepsilon_{ij}\ \ E\ \varepsilon_{ij}=0,$$

$$\text{Cov}(\varepsilon_{ij},\varepsilon_{ik})=\sigma_{ij},$$

where

EPO_{ij} = EPO concentration for the i^{th} child at visit j,
a_{ij} = age of the i^{th} child at visit j,
BPb_{ij} = BPb for the i^{th} child at visit j,
Hgb_{j} = average of the j^{th} child's Hgb at visits 1,2,and 3, and
$I(\bullet)$ = indicator variable taking the values of either 0 or 1.

The results of the model are shown below:

Table A1. Regression coefficient estimates and standard errors (SE)

Variables	Coefficient	Estimate	SE	p-Value
Age 4.5	(α_0)	1.3500	0.1658	<0.0001
Age 6.5	(α_1)	1.6467	0.1748	<0.0001
Age 9.5	(α_2)	1.7453	0.1852	<0.0001
BPb age 4.5	(β_0)	0.2105	0.0421	<0.0001
BPb age 6.5	(β_1)	0.1331	0.0392	0.0007
BPb age 9.5	(β_2)	0.0450	0.0324	0.1645
Hgb	(γ)	-0.0696	0.0135	<0.0001

Table A2. Difference in regression coefficients between two ages

Variables	Difference in coefficient	Estimate	SE	p-Value
Age 6.5 vs. 4.5	$(\alpha_1-\alpha_0)$	0.2967	0.0569	<0.0001
Age 9.5 vs. 4.5	$(\alpha_2-\alpha_0)$	0.3953	0.0564	<0.0001
BPb age 6.5 vs. 4.5	$(\beta_1-\beta_0)$	-0.0773	0.0474	0.1031
BPb age 9.5 vs. 4.5	$(\beta_2-\beta_0)$	-0.1654	0.0480	0.0006

The results of this analysis are consistent with the simple regression models presented in the main body of the paper. EPO appears to increase with increasing BPb at ages 4.5 and 6.5 years, but that relationship flattens out at age 9.5 years. Moreover, the regression coefficients at ages 4.5 and 9.5 years are significantly different, suggesting a true difference in the effect of BPb at these ages.

intoxication: the molecular basis for difference in cutaneous photosensitivity. I. Difference rates of disappearance of protoporphyrin from the erythrocytes, both *in vivo* and *in vitro*. J Clin Invest 56:1519–1527 (1975).
6. Piomelli S, Seaman C, Zullow D, Curran A, Davidow B. Threshold for lead damage to heme synthesis in urban children. Proc Natl Acad Sci USA 79:3335–3339 (1982).
7. Paglia DE, Valentine WN, Dahlgren JG. Effects of low-level lead exposure on pyrimidine-5′-nucleosidase and other erythrocyte enzymes. Possible role of pyrimidine-5′nucleotidase in the pathogenesis of lead-induced anemia. J Clin Invest 56:1164–1169 (1975).
8. Berk PD, Tschudy DP, Shepley LA, Waggoner JG, Berlin NI. Hematologic and biochemical studies in a case of lead poisoning. Am J Med 48:137–144 (1970).
9. Leikin S, Eng G. Erythrokinetic studies of the anemia of lead poisoning. Pediatrics 31:996–1002 (1963).
10. Hernberg S, Nurminen M, Hasan J. Non-random shortening of red cell survival times in men exposed to lead. Environ Res 1:247–261 (1967).
11. Grandjean P, Jensen BM, Sando SH, Jogensen PJ, Antonsen S. Delayed blood regeneration in lead exposure: an effect on reserve capacity. Am J Public Health 79:1385–1388 (1989).
12. Caro J, Erslev AJ. Biologic and immunologic erythropoietin in extracts from hypoxic whole rat kidneys and their glomerular and tubular fractions. J Lab Clin Med 103:922–931 (1984).
13. Erslev AJ, Caro J. Physiologic and molecular biology of erythropoietin. Med Oncol Tumor Pharmacother 3:159–164 (1986).
14. Graziano JH, Slavkovich V, Factor-Litvak P. Depressed serum erythropoietin in pregnant women with elevated blood lead. Arch Environ Health 46:347–350 (1991).
15. Hu H, Watanabe H, Payton M, Korrick S, Rotnitzky A. The relationship between bone lead and hemoglobin. JAMA 272:1512–1517 (1994).
16. Wasserman G, Graziano JH, Factor-Litvak P, Popovac D, Morina N, Musabegovic A, Vrenezi N, Capuni-Paracka S, Lekic V, Preteni-Redjepi E. Independent effects of lead exposure and iron deficiency on developmental outcome at age 2 years. J Pediatr 121:695–703 (1992).
17. Wasserman G, Graziano JH, Factor-Litvak P, Popovac D, Morina N, Musabegovic A, Vrenezi N, Capuni-Paracka S, Lekic V, Preteni-Redjepi E. Consequences of lead exposure and iron supplementation on childhood development at age 4 years. Neurotoxicol Teratol 16:233–240 (1994).
18. Wasserman GA, Liu X, Lolacono NJ, Factor-Litvak P, Kline JK, Popovac D, Morina N, Musabegovic A, Vrenezi N, Capuni-Paracka S, et al. Lead exposure and intelligence in 7-year-old children: the Yugoslavia prospective study. Environ Health Perspect 105:956–962 (1997).
19. Murphy MJ, Graziano JH, Popovac D, Kline J, Mehmeti A, Factor-Litvak P, Ahmedi G, Shrout P, Rajovic L, Nenezic D. Past pregnancy outcomes among women living in the vicinity of a lead smelter in Kosovo, Yugoslavia. Am J Public Health 80:33–35 (1990).
20. Factor-Litvak P, Graziano JH, Kline J, Popovac D, Mehmeti A, Ahmedi G, Shrout P, Murphy MJ, Gashi E, Haxhiu R. A prospective study of birthweight and length of gestation in a population surrounding a lead smelter in Kosovo, Yugoslavia. Int J Epidemiol 20:722–728 (1991).
21. Fernandez F, Hilligoss D. An improved graphite furnace method for the determination of lead in blood using matrix modification and the L'vov platform. At Spectrosc 3:130–131 (1982).
22. Piomelli S. A micromethod for free erythrocyte porphyrins: the FEP test. J Lab Clin Med 81:932–940 (1973).
23. Quantikine IVD. Human EPO immunoassay (ELISA). #DEP00. Minneapolis, MN: R&D Systems, 1997.
24. Liang K-Y, Zeger SL. Longitudinal data analysis using generalized linear models. Biometrika 73:13–22 (1986).
25. Krafte-Jacobs B, Williams J, Soldin SJ. Plasma erythropoietin reference ranges in children. J Pediatr 126:601–603 (1995).
26. Heubel E. Pathogenese und Symptome der Chronishcen Bleivergiftung. Berlin, 1871.
27. Bouchard F. Preliminary report. Compt rendu soc biol 5th Series:358 (1873).
28. McFadzean AJS, Davis LJ. On the nature and significance of stippling in lead poisoning, with reference to the effect of splenectomy. Q J Med 18:57–74 (1949).
29. Griggs RC. Lead poisoning: hematologic aspects. Prog Hematol 4:117–137 (1964).